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20 Variations exist between fish produced naturally and
21 those which are specifically farmed to meet consumer

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1 demand. One particular difference is a variation in
2 the colour of the flesh of the fish.

3

4 The characteristic pink colour of salmonid flesh is a
5 result of the deposition of naturally occurring
6 carotenoid pigments. Obtaining pigmentation in farmed
7 salmonids which is similar to that seen in wild salmon
8 is a vital aspect of feed production. Currently fish
9 feeds contain either or both of the main synthetic
10 pigments which are commercially available; astaxanthin
11 (Ax) and canthaxanthin (Cx). In several instances,
12 pigment costs contribute to 10-15% of the total cost of
13 fish feed production, compared to pigment flesh
14 deposition efficiencies which rarely exceed 15%. Since
15 fish feed comprises around 50% of the total production
16 costs of farmed fish, 5-7.5% of overall fish production
17 cost can be attributed to the cost of pigment.

18

19 Flesh colour is one of the main criteria used by the
20 consumer when considering the purchase of salmonids and
21 accordingly it is considered by the consumer that the
22 stronger red colour of the flesh which is seen in wild
23 fish is more desirable.

24

25 In an effort to achieve the flesh colour
26 characteristics exhibited by wild fish, pigments are
27 added to the feed given to farmed fish with the intent
28 that the uptake, by ingestion of the pigment, will lead
29 to an associated change in the colour of the flesh.

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1 Pigments are specifically selected such that their
2 uptake will lead to the flesh becoming a red colour.
3 Examples of pigments which induce this are
4 canthaxanthin and astaxanthin.

5

6 Such processes are not limited to fish, as the
7 modification of the colour of naturally produced
8 foodstuffs is a current trend. The aesthetic appeal of
9 the product to the end consumer is enhanced through
10 modification of the feed ingredients to influence the
11 characteristics of the final product, in particular the
12 colour of the product.

13

14 An example of such a process currently known in the art
15 is the alteration of the feed ingredients given to
16 chickens and hens, such that the colour of the yolk of
17 the eggs that are produced is modified from that of the
18 natural colour. The result of this process is that the
19 product has an increased aesthetic appeal which in turn
20 leads to a greater desirability for consumer
21 consumption.

22

23 It is desirable for the flesh of the fish to be altered
24 to any specific requirement which may be set. One such
25 method of altering the fish flesh colour would be
26 through the introduction of pigments into the diet.

27

28 It is an object of the present invention to provide a
29 method for improving the uptake of pigments which are
30 provided in the diet to influence the colour of fish
31 flesh.

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1 According to the present invention there is provided, a
2 method of enhancing the uptake of pigment by fish, the
3 method comprising feeding fish with cholesterol.

4

5 Preferably fish are fed cholesterol and pigment.

6

7 Preferably the cholesterol and/or pigment will be a
8 component of the fish feed.

9

10 Also preferably the cholesterol will be provided in the
11 same medium as the pigment.

12

13 Preferably the cholesterol will be added to the fish
14 feed at a level of between 0.1 to 5 percent.

15

16 Most preferably the cholesterol will be added to the
17 feed at a level of between 1 to 3 percent.

18

19 Preferably, the pigment will lead to a change in flesh
20 colour, plasma pigment levels and flesh pigment levels
21 of fish.

22

23 Preferably the method can be used on Atlantic salmon,
24 rainbow trout, other salmonid species, tropical fish.

25

26 Alternatively, the method may be used on any other fish
27 species where the pigment colour of either the flesh or
28 skin is important.

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1 The invention also provides fish feed comprising
2 cholesterol and pigment.

4 The invention also provides the use of cholesterol to
5 enhance uptake of pigment to fish flesh.

7 Deposition of carotenoids in the fish flesh occurs as a
8 result of several processes; absorption of pigments in
9 the digestive tract, transport of pigment in the blood,
0 retention in the flesh and metabolism of carotenoids.

13 1. Absorption

15 Pigment absorption across the intestinal wall,
16 from the digestive tract to the blood is the
17 initial phase in pigment retention by muscle in
18 salmonids. Since carotenoids are lipid soluble
19 they are most likely to be emulsified in a mixed
20 micelle together with bile, other lipid
21 components, prolipase and lipase during absorption
22 across the gastrointestinal tract (Leger 1985).

The rate of pigment absorption to the blood, following ingestion is fairly slow, compared to the absorption of essential fatty acids and amino acids (Storebakken & No, 1992). Maximum plasma Ax and Cx levels occurred at 24 hours following the force feeding of rainbow trout with a 500mg dose of Ax (March et al 1990, Choubert et al 1987),

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1 carotenoid levels first being detected at 3 hours
2 following feeding.

3

4 2. Blood Transport

5

6 Ax and Cx are largely transported by the high
7 density lipoprotein fraction of plasma in immature
8 rainbow trout (Choubert et al, 1992, 1994).
9 Generally in immature fish, flesh is a major
10 tissue for storing carotenoids (No and
11 Storebakken, 1992).

12

13 3. Deposition/Flesh Retention

14

15 Deposition efficiency of dietary carotenoids in
16 salmonid flesh is in the range 1-18% (Torrissen et
17 al, 1989). Astaxanthin retention efficiency of
18 Rainbow trout was found to be significantly higher
19 than that for canthaxanthin; 11.4% and 7.1%
20 respectively (Storebakken & Choubert 1991). Dose
21 response studies show that the efficiency of
22 deposition declines with increase in dietary level
23 (50 mg/kg in Rainbow trout, Storebakken & No 1992:
24 10 mg/kg in Rainbow trout, Crampton 1995).

25

26 Differences in flesh retention efficiencies
27 between species have been observed, and it is
28 known that rainbow trout (RBT) pigment has a
29 greater efficiency than Atlantic salmon (ATS).

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1 In the muscle of wild salmon (*Oncorhynchus keta*,
2 *O. nerka* & *O. kisutch*) astaxanthin (90% in the
3 free form) and canthaxanthin are bound to
4 actomyosin, probably via weak hydrophobic bonds
5 (Henmi et al 1987). Astaxanthin forms two
6 hydrogen bonds per one β ionone ring, and combines
7 more strongly than canthaxanthin, due to its
8 hydroxyl group (see Henmi et al 1989). The
9 actomyosin of salmon muscle can associate with
10 many kinds of carotenoids and lipids, implying
11 that specificity of binding sites is not a
12 problem, with variation between molecule types
13 relating to the bond strength (Henmi et al 1989).
14 In the skin the majority of astaxanthin is found
15 in the ester form (Torrissen et al 1989).

16
17 4 Metabolism

18
19 Carotenoids and their metabolites have been
20 detected in the tissues of fish up to 96 hours
21 following ingestion of a labelled meal (Guillou et
22 al 1992). Schiedt et al (1989) found idoxanthin
23 to be a metabolite of astaxanthin in ATS flesh -
24 higher levels of idoxanthin were found in
25 experimental fish in indoor tanks of farmed fish
26 in open cages, which suggests that this may be
27 stress related (Al-Khalifa & Simpson 1988).
28 Metabolites of carotenoids are found mainly in the
29 skin, but also in the flesh of sexually maturing
30 fish (Hata & Hata 1975; Scheidt et al 1985).

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1 Schiedt et al (1985) evidenced that astaxanthin
2 could be a precursor to vitamin A in vitamin A-
3 depleted fish. Results of Al-Khalifa & Simpson
4 (1988) showed that astaxanthin was converted to
5 zeaxanthin, but in Vitamin A sufficient RBT it was
6 not converted to Vitamin A₁ and A₂ although fish
7 fed a diet lacking in vitamin A and carotenoids
8 for 30 days and then force fed astaxanthin showed
9 an increase in vitamin A.

10

11 This document suggests that the incorporation of a
12 pigment into the diet, either in combination with the
13 foodstuffs directly, or as a separate entity introduced
14 into the diet such that it will enter the same
15 metabolic pathways as other ingested and absorbed
16 nutrients will also end up as a constituent of the
17 flesh.

18

19 The pigment will lead to a change in the colour of the
20 flesh into which it is incorporated.

21

22 The incorporation of the pigment into the flesh may not
23 be efficient and this document identifies a method of
24 enhancing such pigment uptake.

25

26 The benefits of a method by which the uptake of pigment
27 by the fish is enhanced are wide-ranging and cover both
28 biological and economical aspects.

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1 The addition of pigments such as astaxanthin and
2 canthaxanthin can have a drastic economical effect on
3 the cost of producing fish feed pellets, due to the
4 expensive cost of the pigments. As such a more
5 efficient mechanism of producing the effects of
6 astaxanthin and canthaxanthin may lead to a reduction
7 in the amount that needs to be added to the feed
8 initially.

9

10 Some research has indicated that lipid levels improve
11 pigment absorption for example Choubert et al (1991)
12 found that digestibility of Cx was greatly improved
13 when using a lipid rich diet (14% lipid/dry matter cf
14 4% lipid/dry matter). However, at commercially
15 realistic levels of lipid (24-35%) no differences were
16 found in flesh deposition efficiencies of RBT
17 (Crampton, 1996 internal data).

18

19 Bjerking et al (1997) found no significant effect of
20 dietary protein sources (eg a fish meal against a full
21 fat soyabean meal) in Atlantic salmon fed for 9.5
22 months, on the amount of astaxanthin in the muscle or
23 the visual colour score.

24

25 A study of biological utilisation of carotenoids (α and
26 β -carotene) in rats found that bioavailability of
27 naturally occurring carotenoids was greater than the
28 crystalline form (Tee et al 1996). In addition, Bierer
29 et al (1995) found that in pre-ruminant calves higher
30 serum levels of carotenoids were observed when given

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1 commercial beadlet sources compared to crystalline
2 sources.

3

4 A Patent Application in the name of Finnfeeds
5 International Limited, (WO 9818345 A) claims that the
6 absorption in fish, crustaceans and healthy poultry of
7 pigments present in a non-viscous animal feed is
8 promoted by the presence of a carbohydrase and protease
9 enzyme.

10

11 In studies with young chickens Tyczkowski et al (1989)
12 found that lipids, long chain saturated fatty acids
13 (myristic, palmitic, stearic) and triglyceride,
14 tristtearin, promoted minimal absorption of lutein,
15 whereas the short chain saturated lauric acid promoted
16 the highest absorption. Screening trials have been
17 conducted to try and identify enhancers of pigment
18 uptake that may be added to the feed to improve
19 pigmentation.

20

21 Cholesterol was tested as one of the enhancers, due to
22 its properties as an auxiliary agent in uptake.
23 Cholesterol is an important lipid in some membranes and
24 the plasma membranes of eucaryotic cells are usually
25 rich in cholesterol, this steroid also modulates the
26 fluidity of eukaryotic membranes. Due to these
27 properties cholesterol was identified as a substance
28 with the potential to enhance pigment uptake.

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11

1 Cholesterol is added to the feedstuffs either by means
2 of extruder or via flex coating with a level of
3 addition between 0.5% and 5%. Natural levels of
4 cholesterol in commercial fish feeds (derived mainly
5 from fish oil) are up to approximately 0.5%.

6

7 In the same way that the pigmentation of salmonid
8 flesh, eg Atlantic salmon, Coho salmon, Chinook salmon,
9 Rainbow trout, Arctic charr, is important to the
10 consumer, the skin colour of tropical fishes is also an
11 important quality characteristic. In this way the
12 feedstuffs of the above-mentioned species could be
13 modified in a similar way to effect the colour of flesh
14 and skin, in addition to flesh pigment concentration
15 (mg/kg).

16

17 A series of experiments are described below which look
18 at whether there is an enhancement of pigment uptake in
19 the plasma and flesh when the fish feed is supplemented
20 with varying levels of cholesterol.

21

22 Experiment 1

23

24 Atlantic salmon of mean weight 120g, were fed for a
25 period of 72 hours on one of two diets;

26

27 Diet 1: contains approximately 40ppm of canthaxanthin
28 (Cx)

29 Diet 2: contains 40ppm canthaxanthin (Cx) plus 0.48%
30 (total feed, 3% of the lipid coating phase) of
31 cholesterol.

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1 Cx and cholesterol were added in the coating.

2

3 After feeding, fish were bled via the caudal vein,
4 using heparanised vacutainers, the blood samples were
5 centrifuged on site and the plasma removed and stored
6 frozen. Plasma pigment levels were analysed on HPLC.

7

8 Analysis results for the feeds are shown in Table 1.

9

10 TABLE 1 Cholesterol levels in feeds

11

Fish Feed	Cholesterol addition	% Cholesterol in feed
Uncoated feed	0	0.32
coated feed	0	0.27
coated feed	0.48%	0.53
coated feed	0	0.28
coated feed	0.48%	0.54

12

13

14 TABLE 2 Plasma results for the treatmetns

Replicate	Feed No.	Treatment	Cholesterol level % feed	Feed Cx mg/kg	Plasma Cx μ g/ml mean (STD)
1	1	CR	0	40.51	0.94 (0.5)
2					0.64 (0.4)
1	2	CR + Cholest- erol	0.48	45.67	1.42 (0.57)
2					1.45 (0.96)

15

16 significant differences were observed $p < 0.05$ (T-test)

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1.48
0.57
0.85

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1 CR = carophyll red (commercial formulation of Canthaxanthin)

2

3 The results shown in Table 2 clearly show that the fish
4 fed with cholesterol in feed (Diet 2) shown almost a
5 50% increase in the plasma Cx level compared to the
6 control feed. Additionally this trend is apparent in
7 both replicates of the experiments.

8

9 Experiment 2

10

11 Further experimentation investigating the effect of
12 supplementing dietary cholesterol on astaxanthin and
13 canthaxanthin flesh and plasma levels is described
14 below.

15

16 Atlantic salmon of an initial weight of 0.136Kg were
17 grown for four months in 12 x 3m tanks, supplied with
18 seawater. Fish were fed feeds containing varying
19 levels of cholesterol. (Sigma, C8503, approximately
20 95%). Cholesterol was mixed thoroughly with the oil
21 source and added in the coating (in addition to the
22 pigment preparations of astaxanthin (Ax) and
23 canthaxanthin (Cx)). Soya oil was selected as an oil
24 naturally low in cholesterol and this was the basis for
25 using fish foods with different oil source types and
26 the mixture of oils. Details of dietary cholesterol
27 levels and astaxanthin, canthaxanthin concentrations
28 are given in Table 3.

29

30 At the end of the trial, the fish were weighed, they
31 had their blood removed for pigment analysis, and flesh

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1 samples scored with respect to colour and later
2 analysed for pigment.

4 The results of the experiments are further described
5 with reference to the figures wherein;

7 Figure 1 shows the effect of feed cholesterol
8 level on flesh pigment (Cx) concentration (mg/kg),
9 with each point on the figure representing the
0 mean value of each of the tanks,

Figure 2 shows the levels of the pigment canthaxanthin in fish flesh, when cholesterol is added to the feed effect of feed cholesterol level (mg/kg), wherein each point is the mean value of each tank (the 5 pooled analyses),

18 Figure 3 shows the effect of fed cholesterol level
19 on fillet SalmoFan scores and,

21 Figure 4 shows the effect of feed cholesterol
22 level on Minolta redness (a* value).

Figure 1 shows that the plasma pigment levels show an increase which is correlated with an increase in dietary cholesterol to approximately 1-3%. Any further addition of cholesterol to the feed after this level shows a decline in pigment plasma concentration. Maximum canthaxanthin plasma level values were observed at 3.6 $\mu\text{g/ml}$ (1% feed cholesterol added), compared to control values of 1.5-2 $\mu\text{g/ml}$.

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1 Figure 2 shows the effects on the levels of the pigment
2 canthaxanthin in fish flesh, when cholesterol is added
3 to the feed. Maximum flesh pigment levels of around
4 4.3 mg/kg were observed in the group of fish fed
5 canthaxanthin (which have a feed cholesteraoal level of
6 1.3%), compared to levels of around 3 mg/kg in the
7 control groups. In this size of Atlantic salmon,
8 dietary cholesterol levels (1-4%) caused an increase in
9 flesh pigment levels, this increase ranged from 0.4
10 mg/kg to 1.3 mg/kg.

12 Astaxanthin flesh levels were 2.32 mg/kg for the
13 control fish and 2.76 mg/kg for the fish with a 3.8%
14 cholesterol supplement to their feed. Astaxanthin
15 plasma levels were 0.62 µg/ml for the control and 0.65
16 µg/ml for the fish whose feed was supplemented with
17 3.8% cholesterol.

19 The effect of increasing the overall percentage of
20 cholesterol in feed with respect to the resultant
21 colour of the flesh is shown in Figure 3. The colour
22 is scored using a Roche SalmoFanTM score. This is a
23 tool used in the industry to score fish colour, which
24 was developed by Hoffman la Roche Ltd. The test
25 comprises a set of different coloured plastic mini
26 sheets which combine to form a scale that ranges from
27 20 (pale pink) - 34 (dark red), which are used to
28 compare against the colour of the fish flesh and score
29 them accordingly.
30

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1 Maximum SalmoFan scores were observed with the tanks of
2 fish fed 1-2% cholesterol in the feeds. At higher feed
3 cholesterol levels, a decrease in Roche SalmoFanTM
4 scores was observed (Figure 3). The difference in
5 flesh colour shown by the fish fed diets which had been
6 supplemented with between 1-2% cholesterol related to
7 1-1.5 points advantage on the Roche SalmoFanTM test.
8
9 Further analysis of the flesh colour was carried out
10 using the Minolta evaluation technique. Minolta redness
11 values are shown in Figure 2. The Minolta is a
12 tristimulus colorimeter (Minolta Chroma Meter CR300,
13 Minolta, Japan) which has an 8mm head and a D65 light
14 source. Readings were given for Lightness (L*),
15 Redness (a*) and yellowness (b*), the "L a b" system
16 according to International Commission on Illumination
17 (CIE, 1986). Maximum redness values were observed in
18 the fish fed which had been supplemented with between 1-2%
19 of cholesterol, although the pattern was not as clear
20 as that exhibited by the results of the SalmoFanTM
21 scoring system.
22
23 In conclusion, although the experiments described
24 herein show that the addition of any amount of
25 cholesterol to a fish feed at the level of 0 to 5% can
26 results in an increase in pigment levels in the plasma
27 and flesh, the results indicate that the optimum uptake
28 of pigment by the plasma and deposition in the flesh
29 occurs when the feed contains a cholesterol level of
30 between 1 to 3% of total feed weight.

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1 Table 3:

Feed No	Cholesterol Feed Level (%)	Cholesterol Feed Level (%)	Pigment Type	Dietary Pigment Conc (mg/kg)	Oil Source
1441	Control	0.473	Cantha	55.11	fish oil
1442	Control	0.382	Cantha	44.51	Fish/soya oil
1443	Control	0.305	Cantha	50.94	Soya oil
1444	1	1.258	Cantha	46.66	Fish/soya oil
1445	2	2.186	Cantha	50.09	Fish/soya oil
1446	3	3.142	Cantha	52.39	Fish/soya oil
1661	4	4.001	Cantha	50.82	Fish oil
1662	4	3.936	Cantha	53.47	Fish/soya oil
1663	4	3.802	Cantha	48.62	Soya oil
1664	Control	0.412	Asta	47.47	Fish/soya oil
1665	4	3.803	Asta	44.86	Fish/soya sil